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IMPROVING THE VALUE OF FIELD PEAS FOR HUMAN CONSUMPTION MARKETS

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Final Report

Improving the value of field peas for human consumption markets

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Abstract

Visual quality is one of the major factors determining the market value of field pea (*Pisum sativum* L.). Breeding for improved visual quality of pea seeds is currently a challenging task, mainly because of the complexity and the lack of sound genetic knowledge of the traits. The objectives of this research were to characterize the genetic basis and conduct QTL mapping of four key quality traits (cotyledon bleaching in green pea, greenness in yellow pea, seed shape, and seed dimpling in both green and yellow types). Gene expression profiling to understand the molecular basis of post-harvest cotyledon bleaching in green pea was addressed in this project. Two $F_{5:6}$ recombinant inbred line (RIL) populations (93 lines from Orb X CDC Striker cross, and 120 lines from Alfetta X CDC Bronco cross) were developed and evaluated for the quality traits in two locations in Saskatchewan, Canada in 2006 and 2007. The four quality traits evaluated all displayed a continuous range of expression among the relevant RILs, with moderate to high heritability. Two genetic linkage maps utilizing 201 markers (30 SSR (from Agrogene) and 171 AFLP markers) and 155 markers (21 SSR and 134 AFLP markers) were constructed for Orb X CDC Striker and Alfetta X CDC Bronco populations, respectively. Three major QTLs on linkage group (LG) IV and one QTL on LG V for green cotyledon bleaching resistance were identified. Three QTLs on LG I and IV using Orb X CDC Striker population and one major QTL on LG VII with two additional QTLs on LG VII and I using the Alfetta X CDC Bronco population associated with seed shape were recognized. Three QTLs on LG I and IV associated with seed dimpling were recognized using the Orb X CDC Striker population, while no significant QTLs were detected on the linkage map of Alfetta X CDC Bronco population for this trait. Two major QTLs associated with greenness in yellow pea seeds on LG I and II were recognized. The bleaching resistant cultivar CDC Striker had a slower rate of chlorophyll degradation in cotyledons, and a higher carotenoid to chlorophyll ratio in seed coats, than the bleaching susceptible cultivar Orb when seed samples were exposed to high intensity light. An oligo-nucleotide microarray (Ps6kOLI1) was utilized to study the gene expression patterns to understand the molecular mechanism of bleaching resistance in pea seed coats. The gene expression profiles of the CDC Striker and Orb seed coats were significantly different during the seed developmental stages at 14 and 21 days after flowering (DAF). A significant up-regulation of genes involved in the production and accumulation of secondary metabolites which are responsible for antioxidant properties

in plants tissues such as kaempferol 3-0 glucoside, quercetin 3-0 glucosides, pentunidin 3-0 glucoside, and malvidin 3-0 glucosides, in the seed coats of CDC Striker at 14 and 21 DAF were observed.

Executive Summary

Visual quality is one of the major factors that determine the end-use, and most importantly the market value of harvested field pea. Lack of knowledge about the genetic control of visual quality traits in field pea seeds is one of the main constraints faced by pulse breeders.

The genetic study of these quality traits is a challenging task, mainly because of their quantitative inheritance which is strongly influenced by environmental factors. Such quantitative inheritance is often the result of multiple gene segregation. Identification and characterization of the genetic and environmental control of these traits is vital for plant breeding. Application of Quantitative Traits Loci (QTL) analysis has been used for many crop species as an effective tool to uncover the inheritance of quantitative traits such as agronomic and disease resistance, nutritional quality, and seed appearance (Timmerman-Vaughan et al 2004, Timmerman-Vaughan et al 1996, Tar'an et al 2003, Hyton et al 2004, Abbo et al 2005).

Two $F_{5:6}$ recombinant inbred line (RIL) populations, green cotyledon (Orb X CDC Striker) and yellow cotyledon (Alfetta X CDC Bronco), were evaluated in two locations over two years (2006 and 2007) to study the genetic behavior of these visual quality characteristics. The four quality traits evaluated all displayed a continuous range of expression among the relevant RILs, with moderate to high heritability. Two genetic linkage maps utilizing 201 markers (30 SSR (from Agrogene) and 171 AFLP markers) and 155 markers (21 SSR and 134 AFLP markers) were constructed for Orb X CDC Striker and Alfetta X CDC Bronco populations, respectively. Three major QTLs on linkage group (LG) IV and one QTL on LG V for green cotyledon bleaching resistance were identified. Three QTLs on LG I and IV using Orb X CDC Striker population and one major QTL on LG VII with two additional QTLs on LG VII and I using the Alfetta X CDC Bronco population associated with seed shape were recognized. Three QTLs on LG I and IV associated with seed dimpling were recognized using the Orb X CDC Striker population, while no significant QTLs were detected on the linkage map of Alfetta X CDC Bronco population for this trait. Two major QTLs associated with greenness in yellow pea seeds on LG I and II were recognized. In future research, DNA markers will be developed that will allow selection for these key QTLs. These markers will facilitate breeding pea varieties with improved visual quality for export and domestic markets.

This study revealed that the key factor(s) related to cotyledon bleaching resistance in CDC Striker was located in the seed coat, as both CDC Striker and Orb bleached quickly when exposed to high light intensity after dehulling. The bleaching resistant cultivar CDC Striker had a slower rate of chlorophyll degradation in cotyledons, and a higher carotenoid to chlorophyll ratio in seed coats, than the bleaching susceptible cultivar Orb when seed samples were exposed to high intensity light.

An oligo-nucleotide microarray (Ps6kOLI1) developed under the Grain Legumes Technology Transfer platform (GL-TTP) of the Grain Legumes Integrated project (GLIP) was utilized with the collaboration of Dr. Helge Kuster (University of Bielefeld, Germany) to study the gene expression profiles of pea seed coats during the seed development stages. The gene expression profiles of the CDC Striker and Orb seed coats were significantly different during the seed developmental stages at 14 and 21 days after flowering (DAF). A significant up-regulation of genes involved in the production and accumulation of secondary metabolites which are responsible for antioxidant properties in plants tissues such as kaempferol 3-0 glucoside, quercetin 3-0 glucosides, pentunidin 3-0 glucoside, and malvidin 3-0 glucosides, in the seed coats of CDC Striker at 14 and 21 DAF were observed.

Technical Report

Introduction

Field pea (*Pisum sativum* L.) has been accepted throughout the world as a rich source of vegetable proteins and carbohydrates for human diets, as well as in animal feed formulations. In 2007, Canada accounted for 29% (3.02 million tonnes) of the world total pea production (FAOSTAT data, 2008). Pea production from Saskatchewan alone contributed 74% of the Canadian pea production, followed by Alberta at 21%, and Manitoba at 5% (Statistics Canada, Field Crop Reporting Series, Vol.83, No. 8.). Based on the official grain grading guide of the Canadian Grain Commission (2005), good natural color of seeds is considered one of the key quality factors determining grade. To qualify for the highest market grade of green pea (Canada No. 1) seeds should have a natural green color with less than 2% bleached seeds (seeds with more than one-eighth of the surface of the cotyledon bleached to a yellowish color). For yellow pea, natural yellow color with less than 1% of other cotyledon color, such as green or orange, is the key to qualifying for the highest market grade (Canada No.1). Other than seed color, seed shape (round, as opposed to blocky or angular shape) and seed coat texture (smooth, as opposed to dimpled, or "golf –ball" seed surface) are often considered by pulse crop traders beyond the Canadian Grain Commission grading system for both green and yellow peas. In addition, seed size and uniformity of the seeds also play important roles in field pea trading.

Four QTLs controlling seed weight in pea have been identified by linkage mapping, bulked segregant analysis and selective genotyping using RIL derived from two crosses (Timmerman-Vaughan 1996). Biochemical changes during development of the seed pigments (chlorophyll a and b, violaxanthin, neoxanthin, β-carotene and lutein) in pea, and genetic linkage analysis of the green seed color were assessed by McCallum et al. (1997). Significant differences in pigment accumulation and rate of break-down during seed development and seed maturation between the parental lines (OSU442-15 X Primo) were observed. Four genomic regions controlling green seed color were reported by interval mapping using a linkage map produced from 199 molecular markers, bulked segregant analysis and selective genotyping. McCallum et al. (1997) studied the genetic control of green seed color and bleaching during seed development, in contrast to post-harvest, light-mediated bleaching of cotyledon color under investigation in this study. The environmental effects on pea seed color and retention of green color have not been properly addressed to date. Involvement of at least three genes affecting seed coat and cotyledon color in pea genotypes and cotyledon bleaching resistance were reported (Lamprecht 1959; Dribnenki 1979), but no molecular markers linked to the retention of green seed color have been developed so far to facilitate pea breeding programs. The pigments responsible for green cotyledon color of pea seeds are the chloroplast photosynthetic pigments including chlorophylls, carotenoids and xanthophylls (Marx 1977). Bleaching of green seeds during storage is an external symptom due to intracellular break down of photosynthetic pigments as a result of long term exposure to

bright light. The degradation of chlorophyll by photooxidation has been investigated in several plant species (Eckhardt et al 2004; Sagar et al 1988; Feierabend and Schubert 1978). Carotenoids have an important role in protecting chlorophyll pigments from bleaching caused by high light intensities (Griffiths et al 1955; Anderson and Robertson 1960).

Dimpling of seeds, i.e., small, shallow impressions on the testa, is also considered a key visual quality trait in pea that determines market value. Mechanical and textural characteristics of the testa are the major determinant of the appearance of the seed surface. Pectic polysaccharide domains in cells and tissues of the testa play an important role in maintaining the mechanical properties of developing pea seeds, especially at the later stages of seed development (McCartney and Knox 2002). Involvement of a single gene (*mifo*) controlling the dimpling trait of pea seeds was reported by Lamprecht (1962), however no environmental effects were assessed, nor were user-friendly markers developed. In addition, the genetics of seed shape and the appearance of green seed coats in yellow cotyledon pea have not been fully characterized.
Therefore, the current research was undertaken to study the genetic and environmental control of several quality traits in pea including cotyledon bleaching resistance in green pea, seed color in yellow pea, seed shape, and seed dimpling in both green and yellow types.

Objectives
This study was conducted with the following objectives;

1. To characterize the genetic basis of several visual quality traits affecting the market value of field pea.
2. To identify QTLs controlling the visual quality traits of pea seeds.
3. To understand the molecular and biochemical mechanism of the expression of several visual quality traits in field pea

The benefits to a breeding program include knowledge of the biochemical control, genetic control and environmental effects of key traits associated with visual quality in field pea and the identification of molecular markers linked with the genes controlling these traits. Since genetic markers are not affected by environmental conditions, markers will help breeders maintain the improved quality traits in breeding populations without the difficulties imposed by the need to select under erratic environmental conditions. Four studies were utilized to address the above objectives.

Study I: Characterization of the genetic basis of key visual quality traits in field pea.

Introduction

Since the genetic and environmental control of key visual quality traits of pea proposed in this study are not simple, development of RILs and their evaluation over years and locations was deemed to be the best approach. The development and evaluation of RIL populations to evaluate important commercial traits is an established and widely accepted technique (Lee et al 1996; Tar'an et al 2003) for the estimation of genetic parameters of these traits. Proper understanding of the heritable genetic variability and the genotype X environment interactions are important in planning and implementation of effective breeding programs.

Objectives

The objective of this study was to characterize the genetic basis of four visual quality traits affecting the market value of field. The traits studied were cotyledon bleaching resistance in green pea, seed color in yellow pea, seed shape, and seed dimpling in both green and yellow types.

Hypothesis

All four visual quality traits studied are inherent and controlled by multiple genes and their expression is influenced by environmental factors.

Materials and Methods

Five $F_{5:7}$ recombinant inbred line populations (RILs), segregating for several visual quality traits were developed using single seed descent method (Table 1). Among these five populations, the most informative green pea population consisted of 92 RILs derived from the cross Orb X CDC Striker. The most informative yellow pea population consisted of 121 RILs derived from the cross Alfetta X CDC Bronco. These two populations were selected based on the field evaluation of F_3 populations in 2005. The bulked seeds from the other three populations were stored for future studies.

Seeds of the $F_{5:7}$ RILs and parents were grown in the field for accurate assessment ("phenotyping") of the traits of interest. Field trials were conducted using two replicates and two environments in western Canada (Saskatoon (SPG) and Rosthern, SK) in 2006 and 2007. These experiments were laid out in simple lattice design with the plot size of 1 m^2 ("micro-plots"). Standard agronomic practices of the CDC pea breeding program were utilized. Agronomic information, including number of days to flower, lodging score, plant height, powdery mildew reaction and number of days to maturity was recorded during the course of these experiments.

Table 1. Populations developed and traits expected to segregate in each population.

Population	Traits	Low visual quality parental line	High visual quality parental line	Cotyledon color	Traits expected to segregate in F2
1	Bleach	Orb	CDC Striker	Green	Bleach, shape, dimple
2	Green coats	CDC Mozart	DS Admiral	Yellow	Seed color, shape, dimple
3	Shape	Espace	CDC Striker	Green	Shape, bleach
4	Shape	CDC0010	DS Admiral	Yellow	Shape, Seed color
5	Dimple	Alfetta	CDC Bronco	Yellow	Dimple, Seed color, shape

Individual micro-plots were harvested manually when they reached 95% pod maturity and threshed individually to avoid cross contamination. Four representative sub-samples (150 g, 50 g, 40 g and 100 seeds) were drawn from the harvested seeds from each plot. These sub-samples were used to characterize the RILs for the visual quality traits as described in Table 2.

In order to assess green cotyledon bleaching resistance in green pea, a 50g sub sample of whole seeds obtained from each plot of the Orb X CDC Striker population and their parental lines were exposed to high intensity light (1100 micro einsteins $m^{-2}s^{-1}$) for 3 weeks in a growth chamber at 23^{0}C and 60% humidity. Green color bleaching of the seeds was evaluated by measuring the color of the seeds as well as cotyledons using a Hunter Lab colorimeter (Hunter Associates Lab Inc., Reston, VA). The color measurements of the seeds were measured from the whole seeds and the cotyledon color was measured after mechanical seed coat removal. These measurements were taken before and after exposure to high intensity light. The L, a and b values produced by the Hunter Lab colorimeter correspond to the ganglion cells of the human eye sense about the lightness, redness-greenness and yellowness-blueness, respectively (Marcus, 1998). Each sample was scanned using the Hunter Lab colorimeter three times and the calculated average values were used in the data analysis.

In addition, to exposing whole seeds to high intensity light, cotyledon samples, after mechanical seed coat removal, were also subjected to these accelerated bleaching conditions and scanned using the Hunter Lab colorimeter. This step was performed to investigate the effect of seed coats in protecting the green cotyledons from light mediated bleaching.

Seed shape of the both populations was determined by estimating the percentage of round seeds in a 150 g seed sub sample from each plot. The percentage of round seeds was estimated by separating the seed sub-sample using a spherical-nonspherical seed sorter. The average of three readings for each sample was used in the data analysis.

Seed color, especially the remaining green color on the seeds of yellow pea samples is one of the main factors downgrading pea when considered for human consumption markets. The yellow pea RIL population derived from the Alfetta X CDC Bronco cross was utilized to study this trait. Seeds harvested from the field experiments were scanned using Hunter Lab colorimeter. In addition, samples were visually assessed on a 1 to 5 scale, where 1=bright yellow color seeds, and 5=greenish seeds.

Characterization of the seeds for dimpling was done by assigning visual ratings from 1 to 5 (Table 2). Both the presence of dimples as well as the intensity of the dimpling was considered when assigning visual ratings.

Statistical analysis was performed using the proc MIXED procedure of SAS (SAS Institute, Inc., 1997). Genotypes were considered as fixed effects, whereas year, location, replicates and incomplete blocks were considered random effects for the estimation of means for each RIL and the parental cultivars. The parental cultivars were removed from the data sets to facilitate the estimation of variance components of the RILs without confounding them. The genotypes, locations, years, replicates and their interactions were considered random for the estimation of variance components to estimate the genetic parameters. The phenotypic variance was estimated as $\sigma^2_P = \sigma^2_G + (\sigma^2_{GY}/y) + (\sigma^2_{GL}/l) + (\sigma^2_{GLY}/ly) + (\sigma^2_e/lyr)$, where σ^2_G was the estimated genotypic variance, σ^2_{GY} was the genotype year interaction, σ^2_{GL} was the genotype location interaction, σ^2_{GYL} was the genotype year and location interaction, y was the number of years tested, l was the number of locations, and r was the number of replicates per each location. Heritability estimates for each trait were estimated as $H = \sigma^2_G/\sigma^2_P$. Phenotypic correlations and the histograms were constructed using the procedure CORR and UNIVARIATE, respectively.

Results and discussion

Based on the variation observed in the 2005 field experiments for the four key quality traits under investigation, the two most informative populations, Orb X CDC Striker and Alfetta X CDC Bronco were selected for this study (results not shown). Significant variability among the RILs for both populations was observed ($p \leq 0.001$) for all the traits studied within the experiments conducted in 2006 and 2007 at both locations.

Genetics of seed shape (roundness)

A significant high correlation ($r = 88.4$; $P \leq 0.001$) was observed between the visual ratings for the seed shape and the percentage of round seeds estimated by the spherical_nonspherical seed sorter, based on F_3 family evaluation experiments in 2005. This indicated that the estimation of the percentage of round seeds by the spherical_nonspherical seed sorter can be used as an acceptable quantitative way of characterizing the seed shape and was adapted in 2006 and 2007 RIL evaluation experiments.

Analysis of variances of RIL evaluation experiments indicated that the genotypic differences for seed shape measured by the percentage of round seeds were significant ($P \leq 0.0001$) for both RIL populations (Table 3). There was a significant ($P \leq 0.05$) genotype x year interaction for the Alfetta X CDC Bronco population, whereas for the Orb X CDC Striker population it was not significant. For both populations, the genotype x location interaction was not significant ($p \leq 0.05$). However, the genotype x year x location interaction was significant for both populations ($P \leq 0.05$). This indicated that the seed shape was influenced by the environment. Frequency distributions of the percentage of round seeds are shown in Fig. 1.

The seed shape showed continuous normal distribution indicating polygenic control of this trait and quantitative inheritance. The estimated percentage of round seeds for the parental cultivars Orb (57.7) and CDC Striker (75.5) were significantly different ($p \leq 0.05$). This indicated that the seeds of the phenotypically more desirable pea cultivar,

CDC Striker were rounder in shape than Orb seeds. Whereas for Alfetta and CDC Bronco, the seed shape was not significantly different ($p \leq 0.05$) and the estimates of round seed percentages were 76.3 and 75.7 respectively. This indicated that both Alfetta and CDC Bronco had round seed shape. The broad-sense heritability estimates for seed shape were 0.90 and 0.86 for the Orb X CDC Striker population and the Alfetta X CDC Bronco population, respectively (Table 4). The estimated heritability values provided further evidence that this trait is mostly governed by genetic factors with some environmental influences. These results indicated that seed shape can be improved by selection in early generations of a breeding program.

Table 2. Assessment of mapping populations for visual quality traits

Trait	Methods
Cotyledon bleaching resistance in green pea (RIL population derived from Orb X CDC Striker cross)	Color measurements of the seeds and cotyledons* before and after exposure to light were taken using a Hunter Lab colorimeter** utilizing the 50g and 40g sub samples.
Seed color in yellow pea seed (RIL population derived from Alfetta X CDC Bronco)	A. Visual assessments*** B. Measurement of the color of whole seeds (50 g sub sample) using a Hunter Lab colorimeter C. Measurement of the color of the cotyledons* of the 40 g sub sample.
Shape (Both RIL populations)	The percentage of round vs. blocky seeds was determined by separating a 150 g sub-sample of seeds using a spherical-nonspherical seed sorter****
Dimpling (Both RIL populations)	Visual assessments (rating for overall appearance with respect to dimpling*****)

* Seed coats were removed using a Sataki mill to expose cotyledons for color readings

** Hunter Lab colorimeter L, a and b values related to the lightness, redness-greenness and yellowness to blueness of the sample

*** Visual ratings used to evaluate the seed color of the yellow pea seeds
1=100% seeds had bright yellow appearance
2=more than 90% of the seeds had bright yellow appearance
3=more than70% of the seeds were slightly greenish
4=more than 70% of the seeds were moderately greenish
5=More than 95% of the seeds were greenish

**** Seed shape: round/blocky ratio estimated by taking the average ratio of the weight of round seeds to the weight of blocky seeds separated by a spherical-nonspherical (mean of three separations)

***** Visual ratings for seed dimpling
1=more than 95% seeds had a smooth surface
2=around 10% of the seeds were lightly dimpled
3=around 30% of the seeds were lightly to moderately dimpled
4=around 50% of the seeds were moderately to severely dimpled
5=more than 95% of the seeds were severely dimpled

Table 3. Analysis of variances for seed shape (% of round seeds)

a. Orb X CDC Striker RIL population

Source of variation	df	MS
Year	1	3700.6^{NS}
Location	1	0.5^{NS}
Year * Location	1	2491.5*
Replicate (Year * Location)	4	167.9*
Blocks (Year * Location * Replicate)	72	34.7^{NS}
Genotype	89	355.3***
Genotype * Year	89	39.4^{NS}
Genotype * Location	89	33.4^{NS}
Genotype * Year * Location	89	42.1***
Error	272	26.9

CV% = 7.2

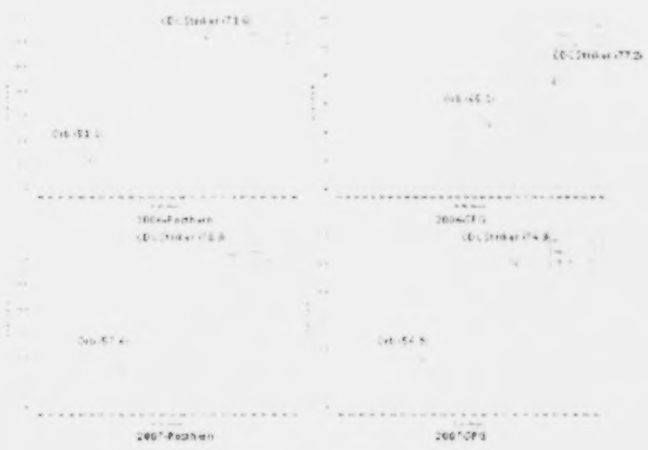
b. Alfetta X CDC Bronco RIL population

Source of variation	df	MS
Year	1	8196.9^{NS}
Location	1	1940.2^{NS}
Year * Location	1	2299.5*
Replicate (Year * Location)	4	197.2*
Blocks (Year * Location * Replicate)	88	23.1**
Genotype	119	261.7***
Genotype * Year	119	31.7*
Genotype * Location	119	23.2^{NS}
Genotype * Year * Location	118	21.6**
Error	375	15.2

CV% = 5.4

Figure 1. Frequency distributions of phenotypes for seed shape (% round seeds) in RIL populations derived from the crosses Orb X CDC Striker and Alfetta X CDC Bronco grown at two locations over two years, as assessed by a spherical_nonspherical seed sorter. Phenotypic values of parental lines are shown by arrows.

a. Orb X CDC Striker RIL population



a. Alfetta X CDC Bronco RIL population

Table 4. Estimates of variance components and heritability for seed shape (% round seeds) in RIL populations derived from the crosses Orb X CDC Striker and Alfetta X CDC Bronco grown at two locations over two years, as assessed by a spherical_nonspherical sorter.

Variance component	Orb X CDC Striker	Alfetta X CDC Bronco
σ^2_G	47.69	33.21
σ^2_{GY}	0.00	2.78
σ^2_{GL}	0.00	0.50
σ^2_{GLY}	2.40	3.72
σ^2_e	29.46	16.81
σ^2_P	53.20	35.58
H^2	0.90	0.86

σ^2_G= Genotypic variance, σ^2_{GY} = Genotypic * year interaction variance, σ^2_{GL} = Genotypic * location interaction variance, σ^2_{GLY} = Genotypic * location * year interaction variance, σ^2_e = Error variance, σ^2_P = Phenotypic variance, H^2 = Broad-sense heritability

Genetics of the seed color in yellow peas

The Hunter Lab colorimeter 'L' (lightness) and the 'a' (greenness) values were significantly different for the genotype and genotype X year interactions, and genotype X year X location interaction (Table 5). Genotype X location interaction was significant for the Hunter Lab 'L' value. Variability for the genotype and genotype X year interaction observed for the seed color ratings were significant ($P \leq 0.001$), whereas the other interaction terms with genotypes were not significant. The broad-sense heritability estimates for the Hunter Lab 'L' value, 'a' value estimates and greenness ratings were 0.67, 0.63 and 0.70, respectively (Table 6). This indicated that this population segregated significantly for seed color with respect to the lightness as well as for the greenness of the seeds and moderately influenced by the environment.

The phenotypically more desirable parental cultivar, CDC Bronco had 'L' value of 55, 'a' value of 5.9, and visual rating of 2.2. Whereas, Alfetta values of 56.7, 6.3 and 2.1, respectively. Except for the visual ratings, both 'L' and 'a' values were significantly different between parental lines ($P \leq 0.001$).

Frequency distribution for this trait for both locations and years for Hunter Lab colorimeter 'L' value (lightness), 'a' value (greenness) as well as the visual color ratings are given in Figure 2, 3 and 4. These histograms indicated a continuous variation suggesting quantitative inheritance with polygenic inheritance for seed color in yellow pea. Breeding programs attempting to improve this trait should evaluate breeding lines over several locations and growing seasons prior to the release of cultivars. This study also demonstrated that the identification and pyramiding of genes contributing to the expression of these traits would be an appropriate approach to improve pea cultivars.

Table 5. Analysis of variances for the seed color of the yellow pea (Alfetta X CDC Bronco) RIL population grown at two locations over two years

a. Hunter Lab 'L' value

Source of variation	df	MS
Year	1	86.7^{NS}
Location	1	1.5^{NS}
Year * Location	1	49.0^{NS}
Replicate (Year * Location)	4	7.4^{*}
Blocks (Year * Location * Replicate)	88	5.5^{***}
Genotype	119	5.5^{***}
Genotype * Year	119	1.6^{*}
Genotype * Location	119	1.2^{NS}
Genotype * Year * Location	118	1.3^{*}
Error	375	0.1

CV% = 1.7

b. Hunter Lab 'a' value

Source of variation	df	MS
Year	1	32.8^{NS}
Location	1	18.9^{NS}
Year * Location	1	7.8^{NS}
Replicate (Year * Location)	4	2.1^{***}
Blocks (Year * Location * Replicate)	88	0.2^{***}
Genotype	119	0.9^{***}
Genotype * Year	119	0.3^{***}
Genotype * Location	119	0.2^{***}
Genotype * Year * Location	118	0.2^{***}
Error	375	0.1

CV% = 5.9

c. Visual rating for greenness

Source of variation	df	MS
Year	1	8.7^{NS}
Location	1	2.9^{NS}
Year * Location	1	46.7^{***}
Replicate (Year * Location)	4	0.3^{NS}
Blocks (Year * Location * Replicate)	88	0.6^{NS}
Genotype	119	2.6^{***}
Genotype * Year	119	0.7^{NS}
Genotype * Location	119	0.5^{NS}
Genotype * Year * Location	118	0.6^{NS}
Error	375	0.5

CV% = 28.3

Table 6. Estimates of variance components and heritability for greenness of the yellow pea seeds in the RIL population derived from the cross Alfetta X CDC Bronco grown at two locations over two years as assessed by Hunter Lab colorimeter 'a' value and visual ratings for the greenness of the seeds.

Variance component	Hunter Lab 'L' value	Hunter Lab 'a' value	Visual rating for seed greenness
σ^2_G	0.58	0.09	0.26
σ^2_{GY}	0.18	0.03	0.04
σ^2_{GL}	0.00	0.01	0.00
σ^2_{GLY}	0.08	0.04	0.01
σ^2_e	0.02	0.12	0.54
σ^2_P	0.86	0.14	0.37
H^2	0.67	0.63	0.70

σ^2_G= Genotypic variance, σ^2_{GY} = Genotypic * year interaction variance, σ^2_{GL} = Genotypic * location interaction variance, σ^2_{GLY} = Genotypic * location * year interaction variance, σ^2_e = Error variance, σ^2_P = Phenotypic variance, H^2 = Broad-sense heritability

Figure 2. Frequency distributions of the lightness of yellow pea (Hunter Lab colorimeter 'L' value) in the RIL population derived from the cross Alfetta X CDC Bronco grown at two locations over two years. Phenotypic values of the parental lines are shown by arrows.

Figure 3. Frequency distributions of the greenness of yellow pea (Hunter Lab colorimeter 'a' value) in the RIL population derived from the cross Alfetta X CDC Bronco grown at two locations over two years. Phenotypic values of the parental lines are shown by arrows.

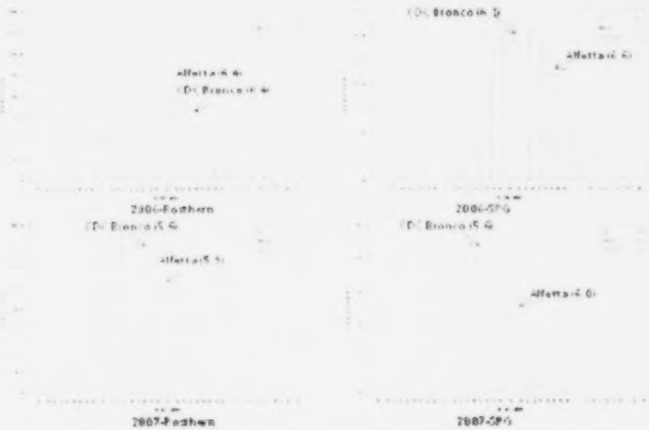
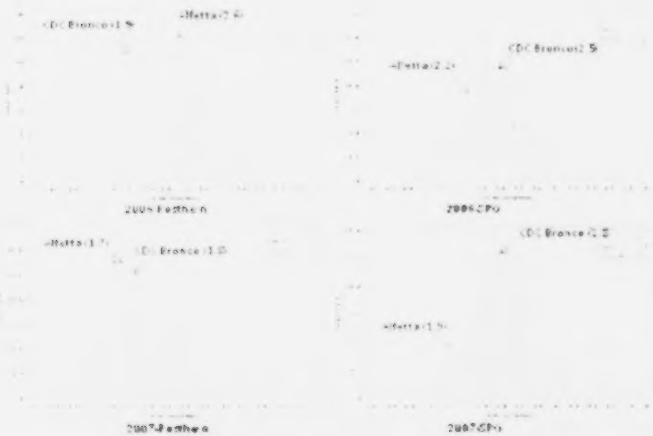


Figure 4. Frequency distributions of the visual ratings for the seed color in yellow pea in the RIL population derived from the cross Alfetta X CDC Bronco grown at two locations over two years. Phenotypic values of the parental lines are shown by arrows. Refer to Table 2 for the rating scale used.



Genetics of cotyledon bleaching resistance in green peas

Seeds and cotyledons of the RIL population from the Orb x CDC Striker population were screened using the Hunter Lab colorimeter L, a and b values before and after exposure to high intensity light for three weeks. A significant ($P \leq 0.001$) genotype and genotype x year interaction was observed for the whole seed greenness before exposure to light (Table 7a). The greenness of the cotyledons measured before exposure to light also showed significant genotype, genotype x year interaction, genotype x location interaction and genotype x location x year interaction terms (Table 7b). The broad-sense heritability of the greenness of whole seeds before exposure to light was 0.72 (Table 8), and the broad-sense heritability of the greenness of the cotyledons was 0.69. This indicates that the phenotypic variation observed for the greenness of the seeds at harvest (whole seeds and cotyledons) is influenced by environmental factors as well as by the genotype x environmental interactions. Frequency distribution of the Hunter Lab 'a' value measured before exposure to light highlighting the cotyledon bleaching resistance in the RIL population is shown in Fig. 5 and 6. This clearly indicated that the green color bleaching resistance was quantitative and under polygenic control.

Seeds were exposed to high intensity light in order to accelerate the green cotyledon bleaching, and therefore evaluate the RILs more effectively by eliminating the environmental variability during the seed bleaching process under field conditions. Greenness of the seeds measured after exposure to light for three weeks showed a significant variation among the RILs (Table 7c). There were no significant differences observed for the genotype x location interaction. However the genotype x year and the genotype x year x location interaction were significant. A similar trend was observed for the Hunter Lab colorimeter 'a' value for the cotyledons after exposure to light (Table 7a). The observed high heritability values of 0.83 for the greenness of the seeds and 0.82 for the greenness of the cotyledons indicated that the genetic contribution for green color bleaching is high (Table 8). Frequency distribution of the Hunter Lab 'a' value measured in seeds and cotyledons after exposure to light are shown in Fig. 7 and 8. These distributions are continuous and skewed toward to the more bleaching resistant parent suggesting most of the alleles contributing to bleaching resistance were from CDC Striker. These results lead to the suggestion of the involvement of few genes with major effects for the control of bleaching resistance.

In contrast to the situation in which seeds exposed to light for 3 weeks had significant genotypic differences in bleaching resistance, cotyledons without seed coats of all RILs and parents lost their green color almost completely after 7 days of exposure to light. Hunter Lab colorimeter 'a' values for all the bleached cotyledons from this RIL population were positive values (Fig. 9). Comparing the phenotypic variability of the RILs for their ability to protect green color with and without seed coats, suggests that the seed coat characteristics of the genotypes play a significant role in bleaching resistance in green peas.

Table 7. Analysis of variance of the greenness (Hunter Lab colorimeter 'a' values) of the seeds harvested from Orb X CDC Striker RIL population grown at two locations over two years.

a. Hunter Lab 'a' values of the whole seeds before exposure to light

Source of variation	df	MS
Year	1	3.54^{NS}
Location	1	0.32^{NS}
Year * Location	1	0.47^{NS}
Replicate (Year * Location)	4	0.08^{NS}
Blocks (Year * Location * Replicate)	72	0.06^{*}
Genotype	89	0.50^{***}
Genotype * Year	89	0.14^{**}
Genotype * Location	89	0.07^{NS}
Genotype * Year * Location	89	0.07^{**}
Error	272	0.05

CV% = 11.6

b. Hunter Lab 'a' values of the cotyledons before exposure to light

Source of variation	df	MS
Year	1	6.94^{NS}
Location	1	0.88^{NS}
Year * Location	1	1.15^{*}
Replicate (Year * Location)	4	0.08^{NS}
Blocks (Year * Location * Replicate)	72	0.08^{*}
Genotype	89	1.24^{***}
Genotype * Year	89	0.35^{***}
Genotype * Location	89	0.20^{*}
Genotype * Year * Location	89	0.20^{**}
Error	272	0.09

CV% = 7.3

c. Hunter Lab 'a' values of the whole seeds after exposure to light

Source of variation	df	MS
Year	1	0.87^{NS}
Location	1	0.02^{NS}
Year * Location	1	0.66^{NS}
Replicate (Year * Location)	4	0.30^{NS}
Blocks (Year * Location * Replicate)	72	0.14^{NS}
Genotype	89	1.55^{***}
Genotype * Year	89	0.27^{**}
Genotype * Location	89	0.13^{NS}
Genotype * Year * Location	89	0.16^{*}
Error	272	0.11

CV% = 25.1

d. Hunter Lab 'a' values of the cotyledons after exposure to light

Source of variation	df	MS
Year	1	13.34^{NS}
Location	1	12.20^{NS}
Year * Location	1	24.16^{NS}
Replicate (Year * Location)	4	2.94^{NS}
Blocks (Year * Location * Replicate)	72	1.56^{**}
Genotype	89	18.43^{***}
Genotype * Year	89	3.37^{**}
Genotype * Location	89	2.02^{NS}
Genotype * Year * Location	89	1.90^{**}
Error	272	0.24

CV% = 15.0

Table 8. Estimates of variance components and heritability for green cotyledon bleaching resistance as measured by the Hunter Lab colorimeter 'a' value for the Orb X CDC Striker population grown at two locations over two years.

Variance component	Before exposure to light		After exposure to light	
	Whole seeds	Cotyledons	Whole seeds	Cotyledons
σ^2_G	0.06	0.16	0.21	0.61
σ^2_{GY}	0.02	0.07	0.04	0.13
σ^2_{GL}	0.00	0.02	0.00	0.01
σ^2_{GLY}	0.02	0.03	0.02	0.10
σ^2_e	0.05	0.09	0.11	0.25
σ^2_P	0.08	0.23	0.25	0.75
H^2	0.72	0.69	0.83	0.82

σ^2_G= Genotypic variance, σ^2_{GY} = Genotypic * year interaction variance, σ^2_{GL} = Genotypic * location interaction variance, σ^2_{GLY} = Genotypic * location * year interaction variance, σ^2_e = Error variance; σ^2_P = Phenotypic variance, H^2 = Broad-sense heritability

Figure 5: Histograms showing the frequency distribution of greenness of the whole seeds (Hunter Lab colorimeter 'a' value) before exposure to light in the Orb X CDC Striker population grown at two locations over two years.

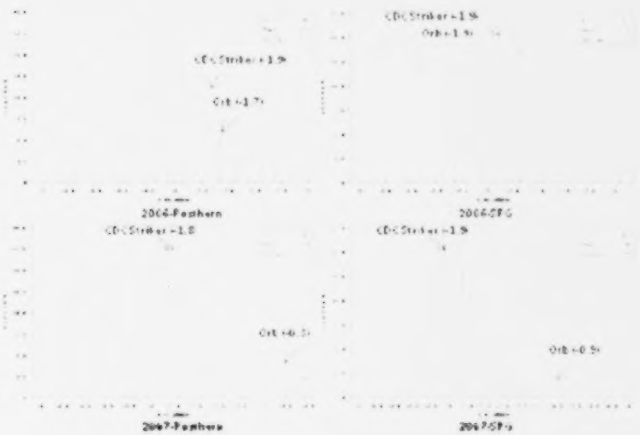


Figure 6: Histograms showing the frequency distribution of greenness of the cotyledons (Hunter Lab colorimeter 'a' value) before exposure to light in the Orb X CDC Striker population grown at two locations over two years.

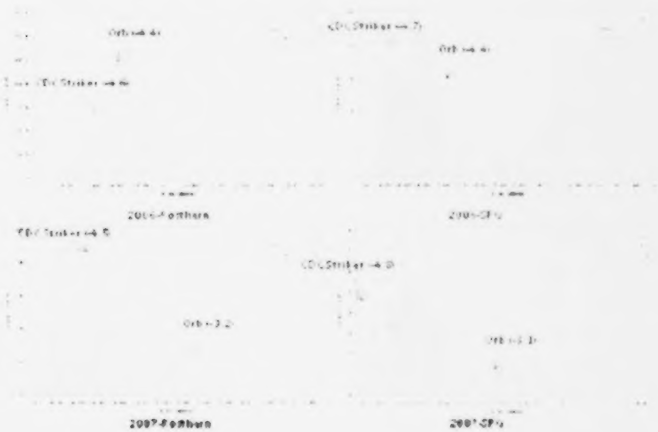


Figure 7: Histograms showing the frequency distribution of greenness of the whole seeds (Hunter Lab colorimeter 'a' value) after exposure to light in Orb X CDC Striker population grown at two locations over two years.

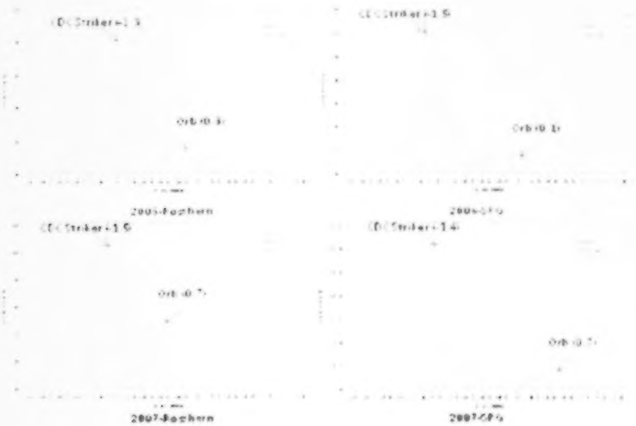


Figure 8: Histograms showing the frequency distribution of greenness of the cotyledons (Hunter Lab colorimeter 'a' value) after exposure to light in Orb X CDC Striker population grown at two locations over two years.

Figure 9: Histograms showing the frequency distribution of greenness of the cotyledons after exposure to light without seed coats (Hunter Lab colorimeter 'a' value) in the Orb X CDC Striker population grown at two locations over two years.

Genetics of seed dimpling

Analysis of variance of seed dimpling indicated that the variability due to genotype and genotype X location X year were significant ($P \leq 0.001$) for both populations (Table 9). The heritability estimates for the Orb X CDC Striker population and the Alfetta X CDC Bronco population for dimpling were 0.73 and 0.86, respectively (Table 10). This clearly indicated that dimpling was highly influenced by genetic factors and moderately by the environmental factors. Thus, selection should be started in the early stages of plant breeding programs and the final variety release should be after intensive screening in multi-location trials in several years.

Visual rating for seed dimpling for the phenotypically superior pea cultivar, CDC Bronco, was 2.0, whereas Alfetta which is more prone to dimpling received a rating of 3.8. These two cultivars were significantly different ($p \leq 0.05$) in their visual ratings. Seed dimpling estimates of the superior parental cultivar (CDC Striker) was 1.5, whereas Orb received a rating of 2.4. Histograms showing the frequency distributions are given in Figure 10 and 11, indicating a clear normal distribution for the dimpling ratings suggesting a polygenic inheritance.

Table 9. Analysis of variances for seed dimpling of two field pea RIL populations grown at two locations over two years (see Table 2 for the description of the visual ratings for seed dimpling).

a. Orb X CDC Striker RIL population

Source of variation	df	MS
Year	1	90.2^{NS}
Location	1	27.7^{NS}
Year * Location	1	18.2^{*}
Replicate (Year * Location)	4	0.5^{NS}
Blocks (Year * Location * Replicate)	72	0.6^{*}
Genotype	89	1.9^{***}
Genotype * Year	89	0.7^{NS}
Genotype * Location	89	0.7^{NS}
Genotype * Year * Location	89	0.5^{*}
Error	272	0.4

CV% = 27.2

b. Alfetta X CDC Bronco RIL population

Source of variation	df	MS
Year	1	9.1^{NS}
Location	1	81.8^{NS}
Year * Location	1	28.3^{*}
Replicate (Year * Location)	4	3.8^{**}
Blocks (Year * Location * Replicate)	88	0.4^{NS}
Genotype	119	4.8^{***}
Genotype * Year	119	0.6^{NS}
Genotype * Location	119	0.5^{NS}
Genotype * Year * Location	118	0.8^{***}
Error	375	0.4

CV% = 23.7

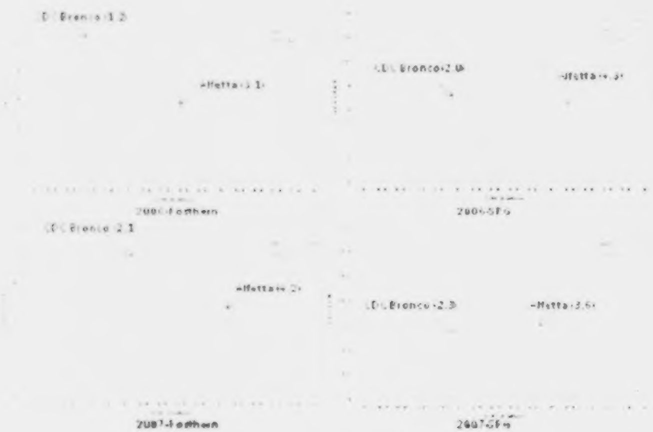
Table 10. Estimates of variance components and heritability for seed dimpling of two field pea RIL populations grown at two locations over two years.

Variance component	Orb X CDC Striker	Alfetta X CDC Bronco
σ^2_G	0.20	0.63
σ^2_{GY}	0.05	0.00
σ^2_{GL}	0.05	0.00
σ^2_{GLY}	0.06	0.12
σ^2_e	0.04	0.44
σ^2_P	0.27	0.74
H^2	0.73	0.86

σ^2_G= Genotypic variance, σ^2_{GY} = Genotypic * year interaction variance, σ^2_{GL} = Genotypic * location interaction variance, σ^2_{GLY} = Genotypic * location * year interaction variance, σ^2_e = Error variance, σ^2_P = Phenotypic variance, H^2 = Broad-sense heritability

Figure 10: Histograms showing the frequency distribution of visual ratings for seed dimpling observed for the RIL population derived from the Orb X CDC Striker cross grown at two locations over two years.

Figure 11: Histograms showing the frequency distribution of visual ratings for seed dimpling observed for the RIL population derived from the Alfetta X CDC Bronco cross grown at two location over two years.



Study II. Genetic mapping and identification of genomic regions controlling several visual quality traits in pea

Introduction

Visual quality characteristics remain as important objectives in pea genetic improvement programs. Evaluating genetic populations for these visual quality traits are challenging as they are influenced by the environment. The progress of conventional breeding programs is considered only moderately effective for these quantitative traits due to the large environmental interactions.

Genetic mapping and identification of genomic regions governing these visual quality traits are important to develop molecular markers which can be used effectively in marker assisted selection with minimal environmental influence.

Objectives

The objectives of this study were to develop genetic linkage maps for the Orb X CDC Striker and Alfetta X CDC Bronco populations and locate the QTLs associated with four visual quality traits, i.e., green cotyledon bleaching resistant, seed color of yellow peas, seed shape and seed dimpling.

Hypothesis

The two RIL populations developed would segregate for the traits of interest and genetic maps could be constructed utilizing molecular marker linkage information. The traits studied in this study are controlled by several regions of the pea genome which could be identified using molecular marker polymorphisms and phenotypic variability of the RILs.

Materials and methods

Leaf samples of 92 and 120 RILs from the Orb X CDC Striker population and Alfetta X CDC Bronco populations, respectively, were collected and freeze dried from field grown $F_{5:7}$ RIL plots at the Saskatoon (SPG) location in 2006 for DNA extraction. DNA was extracted from a 30 g sub-sample of ground freeze dried leaf tissues using a QIAGEN plant mini extraction kit. DNA samples from the parental lines were screened using pea SSR markers (simple sequence repeats or microsatellites) developed by a consortium led by Agrogene, France, to identify informative primer combinations that could be mapped using these two RIL populations. SSR primer pairs were selected if they revealed polymorphism between the parental cultivars. These SSR primer pairs were used to genotype the two mapping populations. In addition, AFLP fingerprinting was employed to genotype the mapping populations. Construction of the genetic map and QTL mapping was performed using JoinMAP® 3.0 (Van Ooijen and Voorrips 2001) and MapQTL® (Van Ooijen 2004) computer software.

Results and discussions

SSR analysis

Three hundred fifty pea SSR markers were screened using the DNA of four parental cultivars (CDC Striker, Orb, Alfetta and CDC Bronco) used to develop the two RIL populations. These preliminary primer screening studies identified 64 and 59 primer pairs with clear polymorphism between Orb/CDC Striker and Alfetta/CDC Bronco, respectively. Of the 64 primer pairs identified for Orb/CDC Striker, only 49 SSR primer pairs yielded segregating loci among the RIL population of Orb X CDC Striker. Fifteen primers initially identified polymorphic between parents had either monomorphic or failed with the RILs. Fifty three loci recognized by these 49 SSR primer pairs were used in the construction of the genetic linkage map of Orb X CDC Striker RIL population.

Fifty nine primer pairs were recognized polymorphic between Alfetta/CDC Bronco and 29 of these were employed to genotype the 94 RIL lines of the Alfetta X CDC Bronco population. Out of these 29, only 24 primer pairs were able to produce segregating SSR loci and 30 of them were utilized in the construction of the genetic linkage map of Alfetta X CDC Bronco population.

AFLP analysis

A total of 274 AFLP loci from 27 primer combinations which were polymorphic between parental cultivars and segregating among the 90 RILs of the Orb X CDC Striker population were scored and used in the construction of the genetic linkage map. Ninety two RILs of the Alfetta X CDC Bronco RIL population were screened using 32 AFLP primer combinations. These primer combinations were able to recognized 274 polymorphic loci between Alfetta and CDC Bronco and among the 92 screened RILs.

Genetic linkage maps

Two genetic linkage maps utilizing 201 markers (30 SSR and 171 AFLP markers) and 155 markers (21 SSR and 134 AFLP markers) were constructed for the Orb X CDC Striker and Alfetta X CDC Bronco populations, respectively (Fig. 12a, 12b and 13). The identified linkage groups (LG) for both populations were anchored to the previously mapped seven linkage groups of the pea genome using the SSR markers as described by Loridon et al. (2005). Fourteen linkage groups representing 5 of the 7 chromosomes in pea genome were recognized in the map derived from Orb X CDC Striker population. Whereas for the Alfetta X CDC Bronco population, 7 linkage groups representing 5 of the 7 chromosomes were identified using the JoinMAP computer software (Van Ooijen and Voorrips 2001). This is due to lack of polymorphic AFLP and SSR markers or insufficient recombination events on LGVI and LGVII of the Orb X CDC Striker population and LGIII and LGV of the Alfetta X CDC Bronco population. Three linkage group of the Orb X CDC Striker map (G3-GROUP3, G1-GROUP5 and G1-GROUP10) were remain unanchored due to lack of anchor markers mapped to these linkage groups.

The total coverage of the Orb X CDC Striker and Alfetta X CDC Bronco maps were 609.8 cM and 297.6 cM respectively.

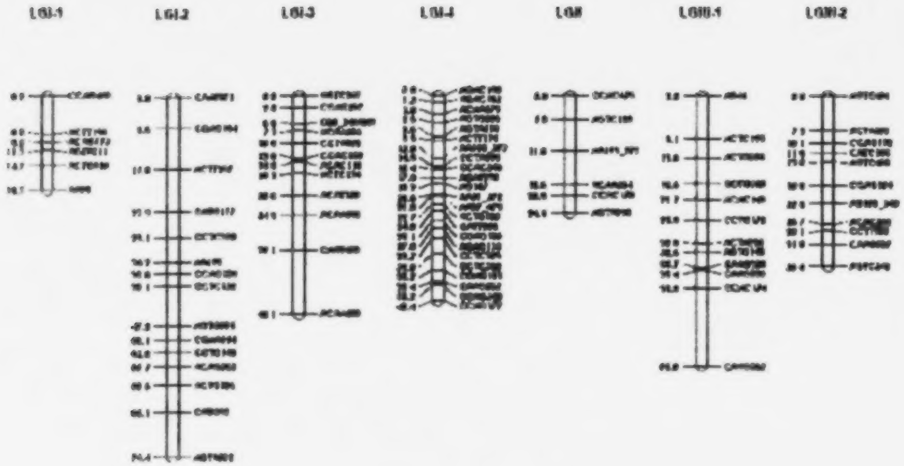


Figure 12a: Linkage map of the pea RIL population derived from the Orb X CDC Striker cross

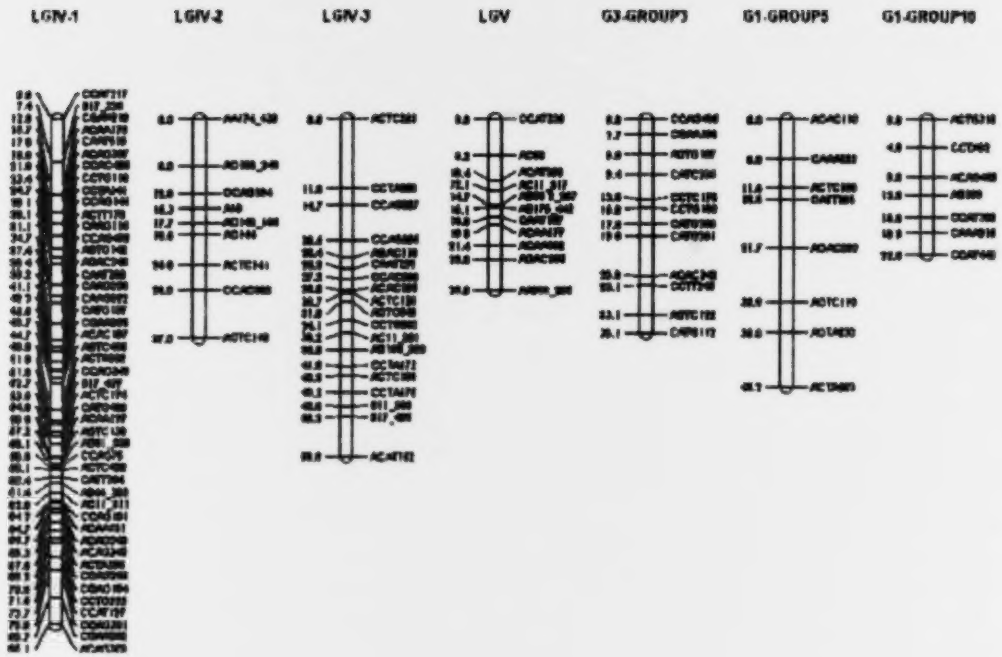


Figure 12b: Linkage map of pea RIL population derived from Orb X CDC Striker cross (continued).
(Linkage groups with no anchor markers to assign previously mapped pea linkage groups by Loridon et al. (2005) are named as G3-Group3, G1-Group5 and G1-Group10)

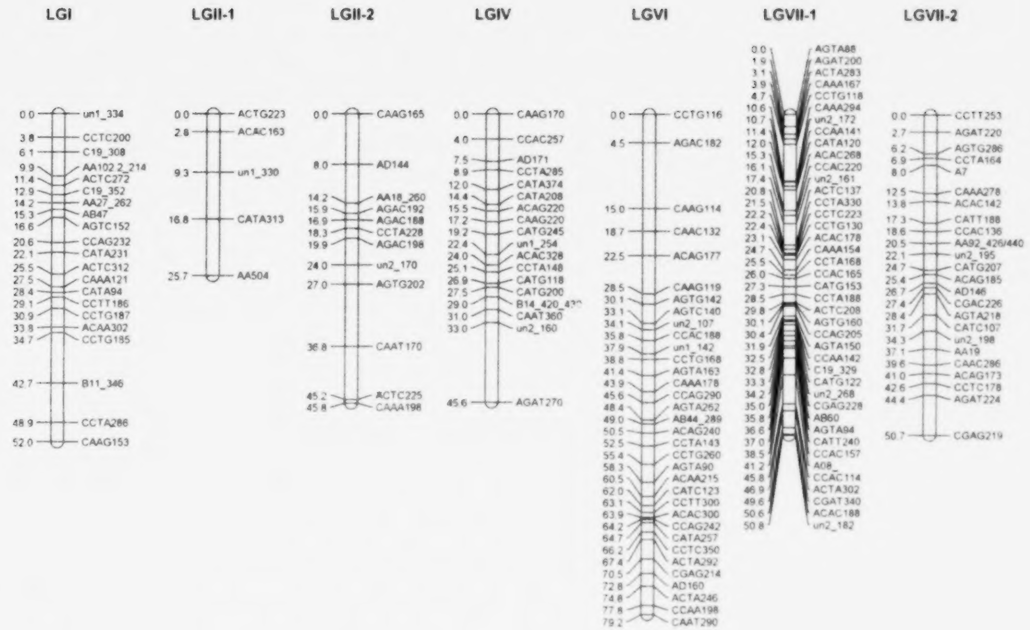


Figure 13: Linkage map of the pea RIL population derived from the Alfetta X CDC Bronco cross

QTL analysis

Putative QTL regions associated with visual quality traits were located by interval mapping and confirmed by the composite interval mapping (MQM) using MapQTL V 4.0 software (Van Ooijen et al 2002). The significant threshold of LOD scores for QTLs detected was determined by permutation test.

Genetics of seed shape (roundness)

Three QTLs on LG I and IV using Orb X CDC Striker population and one major QTL on LG VII with two additional QTLs on LG VII and I using the Alfetta X CDC Bronco population associated with seed shape were recognized.

Genetics of the seed color in yellow peas

Two major QTLs associated with greenness in yellow pea seeds on LG I and II were recognized

Genetics of cotyledon bleaching resistance in green peas

Three major QTLs on LGIV and one QTL on LG V for green cotyledon bleaching resistance were identified.

Genetics of seed dimpling

Three QTLs on LG I and IV associated with seed dimpling were recognized using the Orb X CDC Striker population, while no significant QTLs were detected on the linkage map of Alfetta X CDC Bronco population for this trait.

Study III. Dynamics of the photosynthetic pigments of the seed coat and cotyledons during seed developmental stages and accelerated light mediated cotyledon bleaching in green pea

Introduction

Experiments were conducted to explore the biochemical basis of cotyledon bleaching resistance in green pea. Previous experiment in the current study demonstrated that seed coat characteristics played a significant role in protecting the cotyledons from light induced green color bleaching. Field pea cultivars which contrast for bleaching resistance, Orb (susceptible) and CDC Striker (resistant) were utilized in this analysis. The biochemical basis of green cotyledon bleaching was studied by evaluating the photosynthetic pigment profile changes of the seed coats as well as the cotyledons during seed developmental stages and during light mediated accelerated bleaching conditions after harvest.

Objectives

The objective of this study was to understand the biochemical characteristic of pea seed coats and cotyledons that could be linked to green cotyledon bleaching resistance in green pea.

Hypothesis

Chemical and physical properties of the seed coat determined during seed developmental stages contribute to green cotyledon bleaching resistance properties of different genotypes of green pea.

Materials and Methods

Orb and CDC Striker plants were grown in growth chambers of the University of Saskatchewan phytotron facility. The growing conditions were 23^0C and 17^0C day and night respectively, with photoperiod of 14 hours. Fully opened flowers at the second and third flowering internodes were tagged. Developing pods were collected at 14, 21, 28 and 35 days after flowering. Collected samples were dissected to separate seed coat and cotyledons then frozen in liquid nitrogen and stored at -80^0C until analysis. Seeds samples collected from both parents at 35 days after flowering were placed under artificial light to induce light-mediated post harvest bleaching. Bleaching conditions utilized in this study were the same as for RIL screening experiments for bleaching resistance. Seeds were sampled at 3, 6, 9, 12 and 16 days after exposure to light. Collected seed samples were frozen and stored at -80^0C until analysis after dissecting to separate seed coats and cotyledons.

Pigment analysis

Seed coats and cotyledons from each developmental stage were analyzed separately for pigment profiles (chlorophyll a, chlorophyll b and carotenoids). Extraction and analysis of these pigments were done by spectrometer techniques as described in Sims and Gamon (2002). Differences in pigment profiles were investigated to study the dynamics of the pigment profiles during seed development, and light induced bleaching of mature seeds.

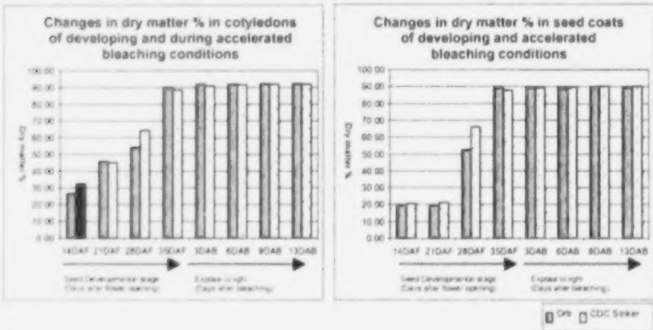
Results and discussions

Dry matter accumulation of the cotyledons and seed coats of Orb and CDC Striker showed a similar trend of increasing during seed filling period and becoming stable at the time of harvesting (Fig. 14a). The only significant variety difference was observed for both cotyledons and seed coats at 28 days after flowering where Orb seed coats had less dry matter (more moisture) than CDC Striker. This graph also indicated that the seed moisture percentage dropped to approximately 10% in both cultivars at 35 days after flowering and remained at this level during the post-harvest light mediated accelerated bleaching period.

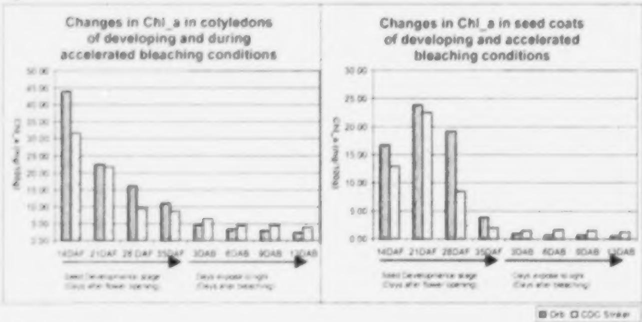
Pigment analysis of the cotyledons and seed coats indicated that the content (mg/100gm dry weight) of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were higher in Orb than CDC Striker until 38 days after flowering (harvesting time under phytotron conditions) (Fig. 14b,c,d and e). However, concentrations of chlorophyll a, chlorophyll b, and carotenoids were higher in CDC Striker than Orb during light mediated bleaching.

The chlorophyll a to b ratio was slightly higher in CDC Striker compared to Orb cotyledons throughout the developmental stages as well as during the light mediated bleaching period (Fig. 14e). During the first 3 days of exposure to light, the pigment profiles in cotyledons and seed coats changed significantly. The rate of chlorophyll degradation in the cotyledons of Orb was apparently higher than in CDC Striker during the time course of this study. The ratios of chlorophyll to carotenoids during the developmental stages up to 35 days after flowering were the same for the both cultivars. However, CDC Striker had a significantly higher carotenoid to chlorophyll ratio than Orb in seed coats after exposing seeds to light for three days, and maintained this higher ratio during the light mediated bleaching period (Fig. 14g).

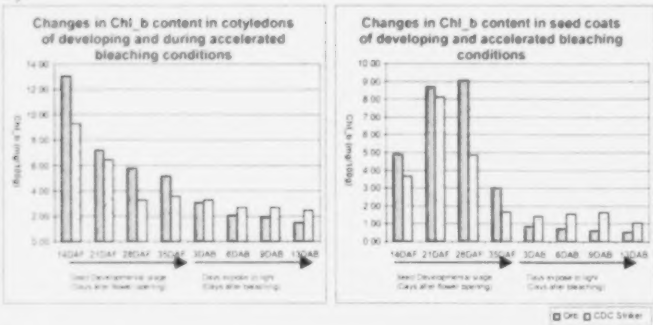
Figure 14. Changes in dry matter, chlorophyll a, chlorophyll b, total chlorophyll, content (mg/100g) chlorophyll a to b ratio, carotenoids, chlorophyll to carotenoids ratio of the seed coats and cotyledons of two parental cultivars (Orb and CDC Striker) during seed development and light mediated bleaching period.



a).



b).



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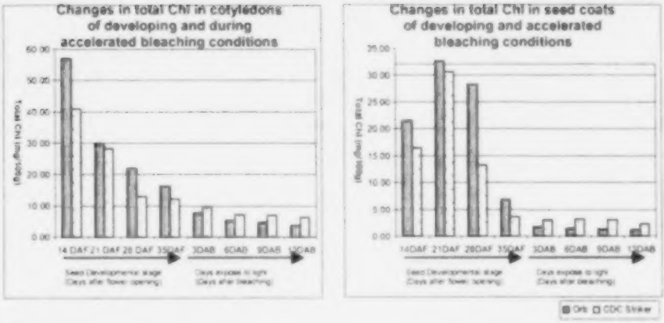
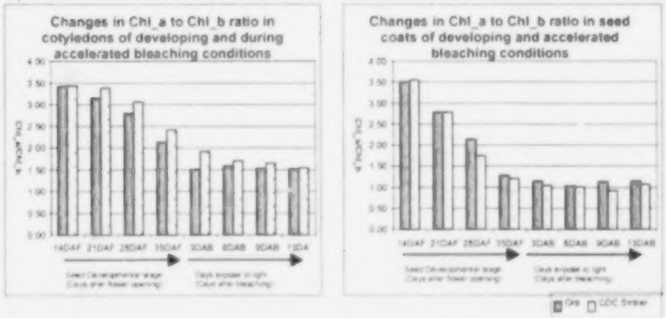
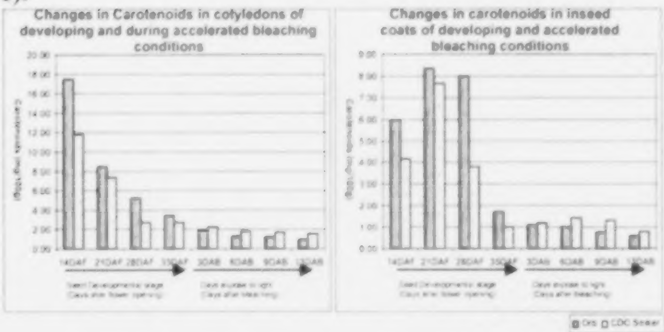

Changes in total Chl in cotyledons of developing and during accelerated bleaching conditions
Changes in total Chl in seed coats of developing and accelerated bleaching conditions
Orb
CDC Striker

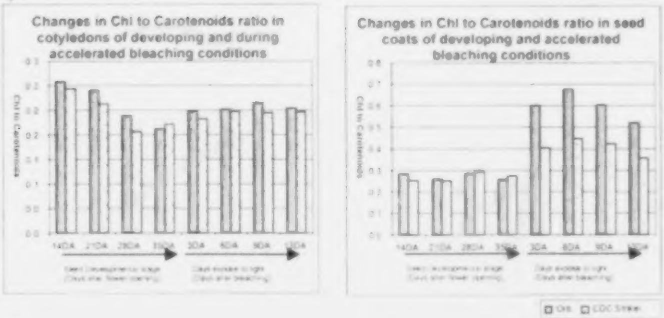
d).


Changes in Chl_a to Chl_b ratio in cotyledons of developing and during accelerated bleaching conditions
Changes in Chl_a to Chl_b ratio in seed coats of developing and accelerated bleaching conditions
Orb
CDC Striker

e).


Changes in Carotenoids in cotyledons of developing and during accelerated bleaching conditions
Changes in carotenoids in inseed coats of developing and accelerated bleaching conditions
Orb
CDC Striker

f).


Changes in Chl to Carotenoids ratio in cotyledons of developing and during accelerated bleaching conditions
Changes in Chl to Carotenoids ratio in seed coats of developing and accelerated bleaching conditions
Orb
CDC Striker

g).

In addition to the pigment profile differences a difference was observed in spectrophotometer UV light absorbance at 326 nm wave length of 80% acetone extracts of seed coat tissues of Orb and CDC Striker (Fig. 15 and 16). UV absorbance at 326 nm was higher in CDC Striker (bleaching resistant) seed coats than in Orb (bleaching susceptible) seed coats (Fig. 15 and 16). These differences were observed between bleached and non-bleached seeds of the same cultivar as well (Fig. 17). These results suggested that a chemical difference of seed coats in CDC Striker may have an association with its bleaching resistance properties compared to Orb.

In order to verify this preliminary observation a follow up research project was conducted using the seed coats of five resistant and susceptible RILs of the population derived from the Orb X CDC Striker population. The results indicated that a significantly different ($p \leq 0.001$) UV absorbance at 326 nm between the seed coat extracts CDC Striker and Orb (Table 11). The difference between resistant 5 RILs and 5 susceptible lines for the UV absorbance at 326 nm was significant ($p \leq 0.001$) and estimated difference was 2.30 with standard error of 0.2. This clearly indicated that the UV absorbance at 326 nm of the seed coat extract was associated with the seed coats of the bleaching resistant. This further confirmed that the chemical composition of the seed coats plays a significant role in bleaching resistance of green pea. Furthermore, identification of the chemical compounds responsible for the high UV absorbance at 326 nm wave length should facilitate a better understanding of the biochemical basis of bleaching resistance in green pea.

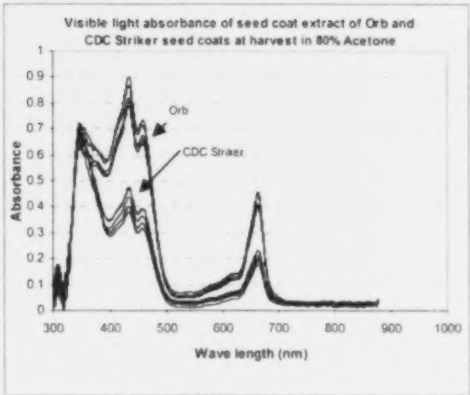


Figure 15. Absorption spectra of acetone extracts of seed coats at harvest (35 days after flowering). Note that the same amount of tissue was used in each extract.

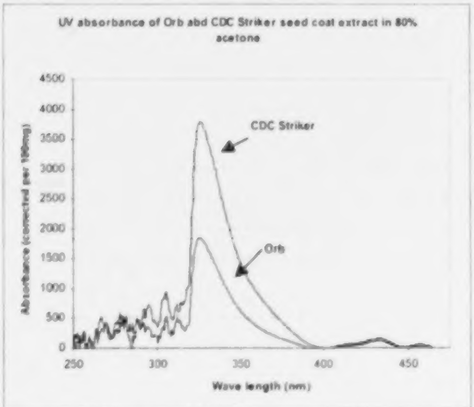


Figure 16. UV Absorption spectra of acetone extracts of seed coats at harvest (35 days after flowering; absorption values corrected for 100 mg of dry matter)

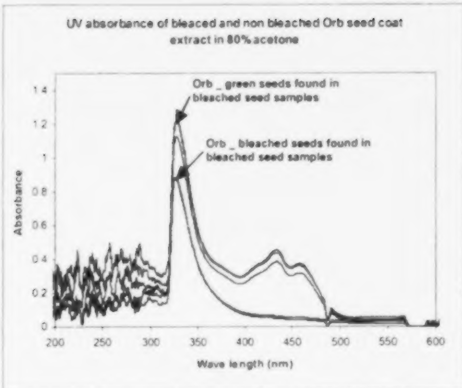


Figure 17. UV Absorption spectra of acetone extracts of seed coats after 16 days of exposure to light. Note that the same amount of tissue was used in each extract.

Table 11. UV absorbance at 326 nm for the seed coat extracts of parental cultivars and selected RILs grown at two locations over two years.

Cultivars and RILs tested		**UV Absorbance at 326nm***
Bleaching resistant	CDC Striker	1.31±0.1
	RIL1-08	1.45±0.1
	RIL1-13	1.59±0.1
	RIL1-50	1.75±0.1
	RIL1-16	1.46±0.1
	RIL1-65	1.61±0.1
Bleaching susceptible	Orb	1.04±0.1
	RIL1-21	1.26±0.1
	RIL1-48	1.20±0.1
	RIL1-35	0.82±0.1
	RIL1-69	0.92±0.1
	RIL1-54	1.14±0.1

*** 1 g of ground seed coat material was used in the extraction with 80% Tris buffered acetone.**

Study IV. Seed coat gene expression profiling of green cotyledon bleaching resistant and susceptible pea cultivars at three developmental stages

Introduction

DNA based microarray analysis provides a platform to study gene expression profiles by the hybridization of two fluorescently labeled cDNA synthesized from two RNA pools simultaneously. The following experimental procedures were conducted to explore the utility of an oligo-nucleotide microarray (Ps6kOLI1) to investigate differences in molecular expression in bleaching resistant (CDC Striker) and bleaching susceptible (Orb) pea cultivars.

This microarray consisted of 4946 non-redundant *Pisum sativum* EST clusters, which originated from various parts of the plants including cotyledons and seed coats at different developmental stages. The Ps6kOLI1 microarray was produced under the framework of the EU FP6 Integrated Project "GRAIN LEGUMES: New strategies to improve grain legumes for food and feed (GLIP)".

Objectives

The objective of this study was to investigate the gene expression profile differences of seed coats at three developmental stages (14, 21 and 28 days) in bleaching resistant (CDC Striker) and bleaching susceptible (Orb) green pea cultivars.

Hypothesis

Green cotyledon bleaching in pea is under genetic control and the gene expression of the seed coats of cultivars which contrast for bleaching resistance is different.

Materials and Methods

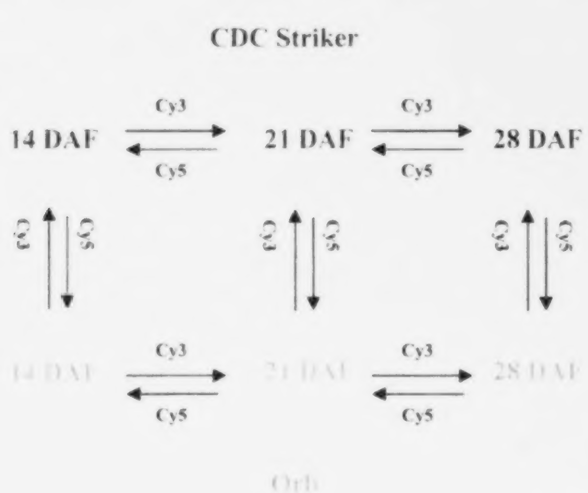
Orb and CDC Striker plants were grown in growth chambers of the University of Saskatchewan phytotron facility. The growing conditions were 23^{0}C and 17^{0}C day and night respectively, with photoperiod of 14 hours. Fully opened flowers at the second and third flowering internodes were tagged at the time of flower opening. Developing pods were collected at 14, 21 and 28 days after flowering. Collected samples were dissected to separate seed coat and cotyledons then ground using mortar and pestle in the presence of liquid nitrogen. Ground tissues were stored at -80^{0}C until RNA extraction.

Microarray hybridization experiments were carried out using the total RNA extracted from the ground seed coat samples collected from Orb and CDC Striker. Total RNA was extracted from seed coats (14, 21 and 28 days after flowering) using the hot acidic phenol method (Chi-Manzanero et al. 2000) and purified using an Invitrogen total RNA extraction kit (Invitrogen Corporation, Carlsbad, CA).

Cy-3 and Cy-5 dye labeled first strand cDNA were synthesized using the extracted RNA from seed coats of parental lines (use one dye to label cDNA strand synthesized from one parent) using CyScribe post labeling kit (GE Healthcare Bio-

Sciences, Uppsala, Sweden). Cy-3 and Cy-5 labeled cDNA were hybridized simultaneously to one microarray in a microarray hybridization chamber at 42^0C for 16 hours. After hybridization, microarray slides were washed, dried and scanned using a GenePix microarray scanner (Molecular Devices, Sunnyvale, CA, U.S.A.). Duplicate hybridizations were performed by swamping the dye labels to eliminate the dye-related signal correlation bias. The complete hybridization scheme is summarized in Fig. 18. Preliminary data analysis using the 1st biological replicate has been completed and briefly presented in this report. Microarray hybridization and scanning of the 2nd biological replicate has been completed and the analysis with both data sets is in progress using EMMA web-based software developed by the CeBiTec, University of Bielefeld, Germany.

Figure 18: Microarray hybridization scheme of seed coat transcriptional profiling



- 14 hybridization steps for one biological replicate
- Two biological replicates were used validation

Results and Discussion

Analysis of 12 microarray slides which were hybridized using Orb and CDC Striker cDNA at different developmental stages using the 1st biological replicate has been completed. The quality of the intensity measurement detected from the slides was found to be within the acceptable range internal replicates and the quality control spots printed on the microarray slides along with the 4946 genes in three replicates.

The gene expression profiles of the two cultivars were significantly different at different developmental stages with respect to the number of up- and down-regulated genes. Based on the genetics and bio-chemical studies conducted on green cotyledon resistance in pea, a few important bio-synthesis pathways which lead to the production of secondary metabolites having antioxidant properties are given in Fig.19, 20 and 21. The green colored histograms on a particular gene indicates a significant up-regulation

($P \leq 0.05$) of CDC Striker seed coats at particular developmental stage compared to Orb seed coats at that stage, while red color indicates down-regulation. These results indicated that the gene responsible for the conversion of p-Coumaric acid to p-Coumaroyl CoA which is the precursor for the flavanoid biosynthesis pathway is significantly up-regulated in CDC Striker seed coats at all three developmental stages studied. Furthermore, the genes responsible for the conversion of p-Coumaric acid to other compounds such as hydroxystyrene and caffeic acid are significantly up-regulated in Orb. These changes indicated that the net synthesis of p-Coumaroyl CoA should be high in CDC Striker seed coats at all three developmental stages compared to Orb. The transcriptional differences observed within the flavanoid biosynthesis pathway are given in Fig 20. This indicated that the genes responsible for the production of a series of secondary metabolites which are responsible for antioxidant properties in plants tissues such as kaempferol 3-0 glucoside, Quercetin 3-0 glucosides, pentunidin 3-0 glucoside, malvidin 3-0 glucosides are significantly up-regulated in seed coats of CDC Striker than Orb at all three developmental stages.

In addition to the transcriptional differences observed for the biosynthesis of secondary metabolites, significant differences in gene expression were detected in the terpenoid pathway (Fig. 21). The activity of the gene responsible for the enzyme which converts geranylgeranyl-PP to prephytoene-pp is up-regulated in the seed coats of CDC Striker at 14 and 21 DAF stages, indicating more precursors for carotenoid biosynthesis than in Orb seed coats.

These results are providing evidence to the existence of transcriptional differences of the seed coats during seed development of bleaching resistant and susceptible cultivars that could be linked to green cotyledon bleaching resistance in pea.

Figure: 19. Differential gene expression pattern within the coumarin and phenylpropanoid biosynthesis pathways of the seed coats of CDC Striker and Orb at 14, 21 and 28 days after flowering. (Yellow boxes indicating that the gene expression responsible for a particular step is significantly different. Green and red histograms representing up- or down-regulation at 28, 21 and 14 DAF in CDC Striker over Orb).

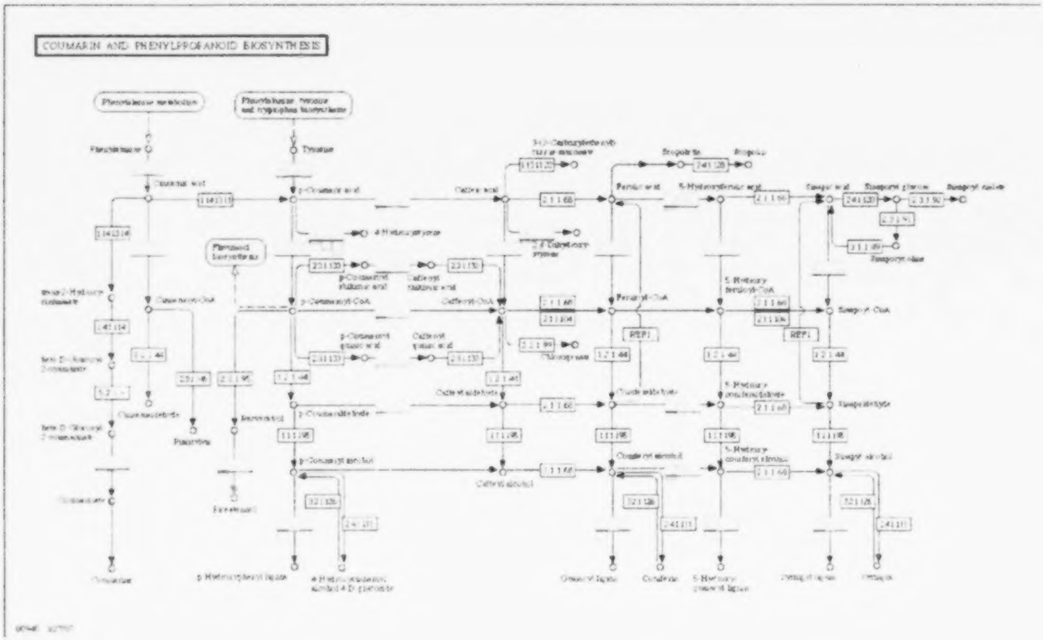


Figure: 20. Differential gene expression pattern within the flavanoid biosynthesis pathway of the seed coats of CDC Striker and Orb at 14, 21 and 28 days after flowering. (Yellow boxes indicating that the gene expression responsible for a particular step is significantly different. Green and red histograms representing the up- or down-regulation at 28, 21 and 14 DAF in CDC Striker over Orb).

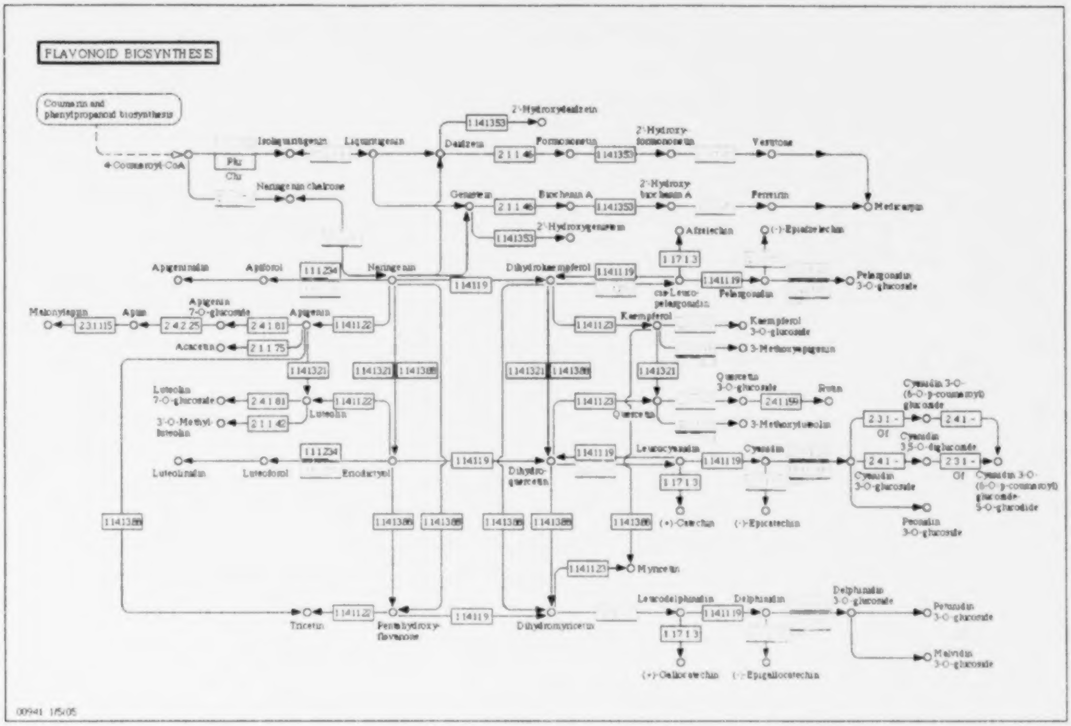
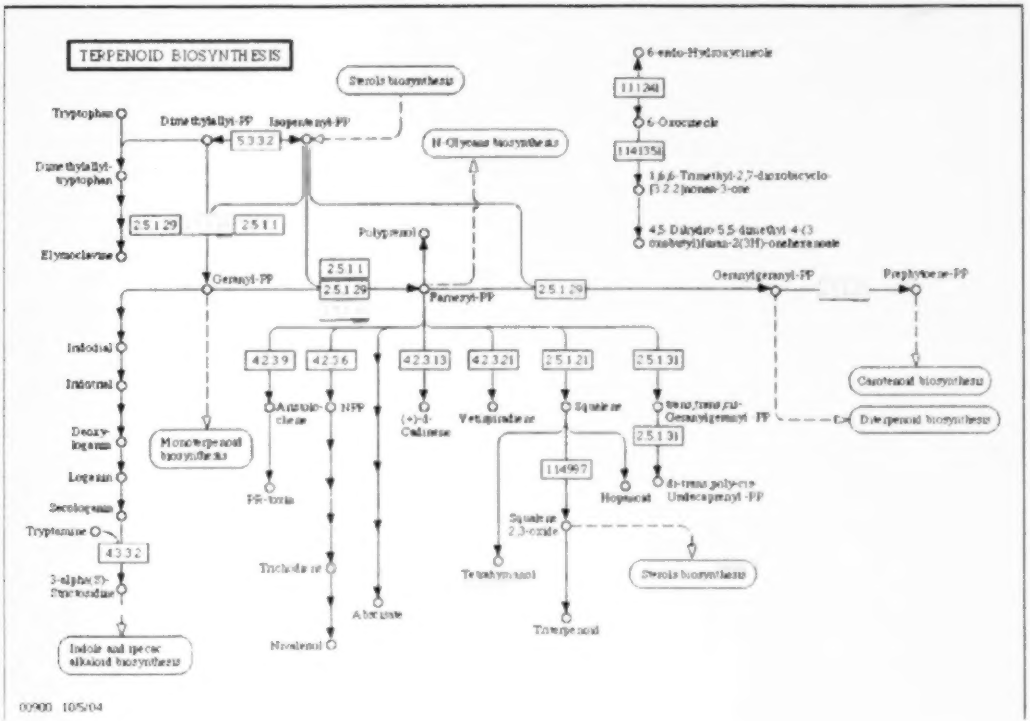


Figure: 21. Differential gene expression pattern within the terpenoid biosynthesis pathway of the seed coats of CDC Striker and Orb at 14, 21 and 28 days after flowering. (Yellow boxes indicating that the gene expression responsible for a particular step is significantly different. Green and red histograms representing the up- or down-regulation at 28, 21 and 14 DAF in CDC Striker over Orb).



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L. Ubayasena, T. Warkentin, K. Bett, B. Tar'an, P. Vijayan and D. Bing. 2009. Identification of genomic regions controlling key visual quality traits affecting the market value of field pea. Pulse Days 2009, Saskatchewan Pulse Growers.

L. Ubayasena, T.D. Warkentin, K. Bett, B. Tar'an, P. Vijayan, and D. Bing, 2008. Genetic Analysis, QTL Mapping and Gene Expression Analysis of Key Visual Quality Traits Affecting the Market Value of Field Pea. Pulse Days 2008, Saskatchewan Pulse Growers.

S. Miller, L. Ubayasena, T. Warkentin, and K. Bett, 2007. The Effect of Light Quality on Green Pea Bleaching. Pulse Days 2007, Saskatchewan Pulse Growers (Saskatoon, SK).

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T.D. Warkentin, K.E. Bett, B. Tar'an, V. Racz, G. Arganosa, L. Ubayasena, H. Classen, and A. Vandenberg, 2005. Improving Field Pea Quality for Food and Feed Markets. North American Pulse Improvement Association Meeting (Newark, Delaware).

Manuscripts in preparation

Ubayasena, L., et al. Genetic control and identification of QTLs associated with bleaching resistance in green field pea.

Ubayasena, L., et al. Genetic control and identification of QTLs associated with seed shape, seed dimpling and greenness in field pea.

Ubayasena, L., et al. Gene expression and biochemical analysis of photosynthetic pigments associated with bleaching in field pea.

Information of benefit to producers, processors, or governments

The genetic control of four visual quality traits in field pea was revealed in this research project. The seed shape of both yellow and green cotyledon pea, dimpling of both yellow and green cotyledon pea, greenness of yellow pea, and green colour bleaching of the cotyledons of green peas are under polygenenic control and display quantitative inheritance. Chromosomal regions (QTLs) were identified which were associated with these key traits. In future research, DNA markers will be developed that will allow selection for these key QTLs. These markers will facilitate breeding pea varieties with improved visual quality for export and domestic markets.

a) Personnel

A portion of the PhD graduate student stipend of Mr. Lasantha Ubayasena was paid from this project fund. A portion of technician Ms. Parvaneh Hashemi's salary was also paid from this project fund.

b) Equipment

No equipment was purchased.

c) Project developed materials

Five RIL ($F_{5\,6}$) populations were developed. Two of these were been advanced to the $F_{5\,8}$ stage. One of these has been advanced to the $F_{5\,7}$ stage.

d) Project photos

Mr. Ubayasena took many photographs of greenhouse and field grown pea populations, as well as DNA gels, which are available on request.

e) Acknowledgement

Saskatchewan Pulse Growers and Saskatchewan Agriculture and Food have been acknowledged in all presentations where this research was discussed.

f) Expense statement

Will be supplied.

g) ICAR Data Entry

Will be entered.